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TONSILS AND APOLIPOPROTEINS:
LESSONS ABOUT PLASMA
LIPOPROTEINS DERIVED FROM
TANGIER DISEASE AND
OTHER MUTANTS

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TONSILS AND APOLIPOPROTEINS: LESSONS ABOUT PLASMA LIPOPROTEINS DERIVED FROM TANGIER DISEASE AND OTHER MUTANTS

It is a great privilege to give this lecture dedicated to Professor Carlos Jiménez-Díaz. To honor the memory of this distinguished physician, who was also a physiologist with considerable interest in fat metabolism, I have chosen a topic that interweaves the study of human disease and the physiology of lipid transport. As do most stories of research, mine relates the slow and often unpredictable manner in which lines of experimental observation converge to form new knowledge. And when I have finished I will have reinforced the axiom that knowledge is invariably incomplete.

My story begins with a child who had strange appearing pharyngeal tonsils, but courses primarily through some highlights of the emergence of understanding of plasma lipoproteins. These macromolecules have developed in higher forms of life for the inter-organ transportation of lipids, substances of little solubility in water. The lipoproteins provide dispersions of lipids that are at once stable and yet easily handled at their destinations. In recent years, research on lipoproteins has tended to concentrate on the protein constituents, the apolipoproteins. I will here be particularly concerned with the structure and function of plasma high density lipoproteins (Fig. 1) and the nature of their apoprotein constituents.

TANGIER DISEASE

In the company of a succession of gifted colleagues I have been engaged for about 20 years in the study of plasma lipoproteins and the diseases that affect them. Much of the last six years has been devoted to isolation of apoproteins and characterization of their structure as groundwork for studies of lipoprotein synthesis and degradation and the regulation of these processes. Quite often we also have taken side-trips into genetically-determined diseases that are not obviously related to plasma lipid transport.

It was on the latter basis that Dr. Paul Altrocchi and I found ourselves one September day in 1960 on a small island in the Chesapeake Bay. Tangier Island (Virginia) was discovered in 1608 by Captain John Smith, the same explorer who had founded Jamestown,

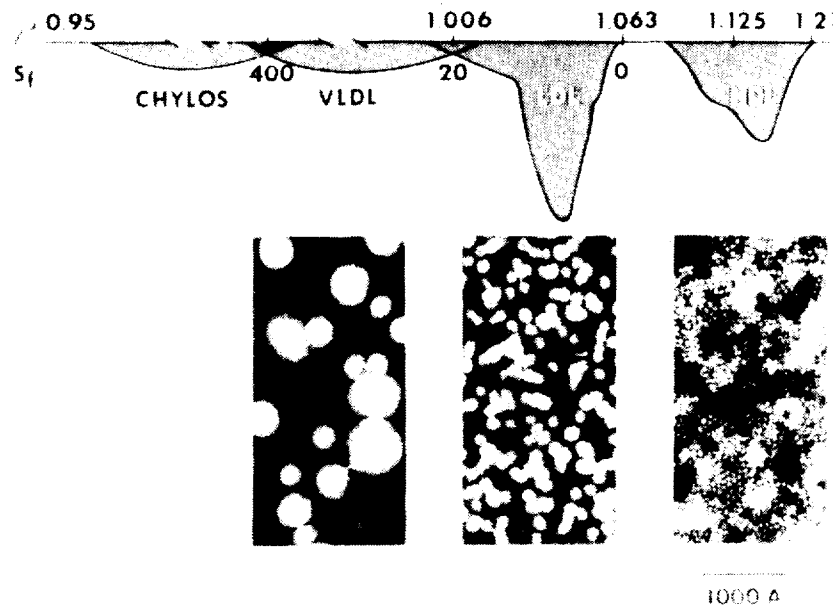


FIG. 1. Schematic representation of the four major plasma lipoprotein families, visualized as Schlieren patterns obtained in the analytical ultracentrifuge, and the appearance of negatively stained VLDL, LDL, and HDL under the electron microscope.

the first English colony in America one year before. Many of the 900 fishermen and their families who live there today are descended from the John Crockett who founded a settlement there in 1686.

The five-year-old son of one of these families had brought us to the island. His tonsils had been removed a few months earlier and were discovered to be loaded with large fat-filled histiocytes (1-6). We had examined the throats of a large number of the island inhabitants, when, at the end of the day, we were presented with the six-year-old sister of our original patient. I still recall vividly the sight of her bright-orange and grotesquely-enlarged tonsils (Fig. 2). The view was accompanied by that sense of exhilaration that a climber feels when he first confronts a new mountain route not yet explored.

In the next few months we had obtained those tonsils and gathered much more information. The tonsils proved to be only the most visible markers of wide spread lipid storage throughout the reticuloendothelial system. Foam cells were present in the bone marrow, liver, lymph nodes, and, as we have learned subsequently, in the thymus, intestinal mucosa, spleen, and other tissues. The substance accumulating was cholesterol, nearly all in the esterified form. It existed as doubly-refractile droplets and probably sometimes as crystals. The quantities that were stored were prodigious, sometimes exceeding the normal tissue cholesteryl ester concentration by more than 100 fold (Table 1).

Experiments made then, and later, revealed no local biochemical defects that explained this sterol accumulation. Synthesis of cholesterol in the abnormal tonsil was brisk, but not obviously greater than in similar unaffected tissue (7). The placement of the lipid deposits in the cytoplasm and unbounded by membranes was the antithesis of the usual lysosomal hydrolase deficiencies (8). The acid cholesteryl ester hydrolase activity in the tissues appeared to be normal. [By chance we had discovered about the same time a cholesteryl



FIG. 2. The tonsils of a patient with Tangier disease. The color is bright orange and similar-appearing tags are sometimes visible in the pharynx after tonsillectomy.

ester storage disease that was due to the deficiency of such an enzyme (9). Beyond similar accumulation of sterol esters, its features had little relationship to the island disease (10).

Preoccupied by the tissue changes, we had ignored at first what now must be considered the principal defect in this new mutant. This lay not in tissues, but in the blood. The plasma cholesterol was abnormally low and the triglycerides in excess (Table 2). Such paradoxical changes in these plasma lipids were superficially explained by analyses of the plasma lipoproteins. It will be hard to forget the first time when we collected from the preparative ultracentrifuge the lipoprotein fraction corresponding to high density lipoproteins (HDL) from two siblings. Unlike any analyses we had ever seen, the HDL fraction was colorless and devoid of Schleiren lines. Ashamed of my apparent mistake in adjusting the density of the plasma, I ran the centrifugal sequence again, and again. The result was unequivocal. Tangier disease—as we had begun to call it—was a familial deficiency of HDL. The cholesterol content of this fraction was persistently 1-5 mg per dl., instead of the usual 40 to 50 mg per dl. (Table 3). By immunochemical analyses, a faint precipitin line was obtained with antiHDL sera (1). We presumed that a little normal HDL was present, but that its synthesis was inadequate or the lipoprotein could not be retained in plasma, perhaps because it was defective.

We returned to Tangier Island and reconstructed the pedigree of the patient's family by interviews with older inhabitants (3). Records were non-existent. The evidence for consanguinity was scanty, the first common relative being detected seven generations removed from our *propositi*. Nevertheless, measurements of HDL concentrations in the parents and other first degree relatives indicated that Tangier disease is caused by a mutation at a locus on autosomal gene. (I may add that this genetic mode has been consistently borne out in studies of 12 kindreds in which Tangier disease has subsequently been found.)

TABLE 1.—*Tonsil Lipids*

	CE	FC	PL
Tangier	136	10	40
Control	2	13	32

(mg/gm dry wgt)

Abbreviations:

FC, free cholesterol; CE, cholesteryl esters; and PL, phospholipids (5).

TABLE 2.—*Plasma Lipids*

	C	TG	PL
Tangier	77	205	101
Control	180	60	200

(mg/100 ml.)

Controls represent mean values for a large sample of children of both sexes, ages 1 to 19 years. The patient with Tangier disease was 8 years old. Samples were obtained after an overnight fast.

Abbreviations: C, cholesterol; TG, triglycerides; and PL, phospholipids.

TABLE 3.—*Lipoprotein concentrations in first two patients with Tangier disease*

	Whole Plasma		D < 1.019		D 1.019 - 1.063		D 1.063 - 1.21		D > 1.21	
Patients	C	PL	C	PL	C	PL	C	PL	C	PL
1.	84	113	53	55	31	42	< 1	5	< 1	11
2.	93	127	35	57	56	56	2	2	< 1	12
Controls	171	215	26	31	97	69	43	111	< 2	21

Abbreviations: C, cholesterol; PL, phospholipids; D, density.

There was ample evidence in our Tangier Island children of involvement of lipoproteins in classes other than HDL. The persistently high triglyceride concentrations were accompanied by aberrations in the lipid composition of low density lipoproteins (LDL). The LDL were not completely absent from the plasma, however, in contrast to abetalipoproteinemia. This is another form of dyslipoproteinemia due to mutation. The characteristic lipoprotein changes in abetalipoproteinemia were discovered in the same year as Tangier disease, 1960, by Salt *et al.* (11) and others (12, 13). There were other differences between Tangier disease and abetalipoproteinemia that were immediately apparent. Tangier

disease appeared to be more benign, lacking the severe malabsorption, acanthocytosis and central nervous system disease of abetalipoproteinemia (6). Many of us became aware of the potential lessons about plasma lipoproteins these mutants held. By comparing them, we concluded that deletion of LDL was more serious than HDL, and therefore the functions of the former lipoproteins must be more important. At the same time, however, we became conscious of how little we know about the functions, and for that matter, the structure and relationships of the several lipoprotein classes.

KNOWLEDGE OF PLASMA LIPOPROTEINS IN 1960

The modern era of lipoprotein research began in the later 1940's, particularly in Boston, with the research of Oncley and others working with Edwin Cohn, and in Berkeley, under the guidance of John Gofman. It was the latter, with Frank Lindgren, who designed the separation techniques and gave us the language of the ultracentrifuge that has been used to describe the lipoproteins for the past 20 years. Framed against an idealized Schleiren pattern (Fig. 1) the major classes are very low density lipoproteins (VLDL) which float at D 1.006, the low density lipoproteins (LDL), separated between D 1.006 and 1.063, and HDL which are obtained between D 1.063 and 1.21. As shown in figure 1 the particle size of the lipoproteins increases as the density decreases. For two to eight hours after the ingestion of fat, chylomicrons, very large particles of the lowest density, also appear transiently in plasma. They cannot be clearly separated from the VLDL, which transport «endogenous» glycerides, arising in the liver and small intestine.

For practical purposes, one may consider these lipoproteins in two functional groups. The chylomicrons and VLDL transport triglycerides, and the LDL seem to represent mainly the end products of this process, or residual vehicles which linger in plasma after the triglycerides and certain other constituents have been removed. HDL contain little glyceride (5 percent by weight) and are made up mainly of protein, phospholipid and cholesterol (Fig. 3). HDL also participate in triglyceride transport, but in a tangential manner that probably does not represent their sole, or even major function.

One suspects that HDL have a role that is quite primitive. In most species, like the rat, dog, horse, and many others, HDL are the predominant lipoprotein class and carry 2/3 or more of the plasma cholesterol (14). The same is true in human fetal plasma but, almost immediately after man is born, triglyceride transport becomes very active, and shortly the combined VLDL and LDL become the more prominent carriers of fats and sterols in the plasma of man (15).

In 1960, information about the structure of HDL was scanty and provided no basis for speculation about the locus of the genetic defect in Tangier disease. This was equally true for all the lipoproteins, particularly their protein moieties or «apolipoproteins» as they came to be called (16, 17). It was believed there were two such proteins, an α (A) and a β (B) that resided, respectively in HDL and LDL, and that the resulting lipid-protein complexes also took on extra glycerides to form chylomicrons and VLDL (Fig. 4). It was a time when a number of workers were beginning to discover how to remove the lipids from lipoproteins without irreversibly denaturing the proteins, and the major tools for recognizing different proteins in a mixture consisted of immunochemical analyses and methods for identifying terminal amino acids.

Our laboratory entered the search for apolipoproteins in the mid-1960's when powerful chromatographic techniques for separating proteins by their size and charge became

COMPOSITION OF HIGH DENSITY LIPOPROTEINS

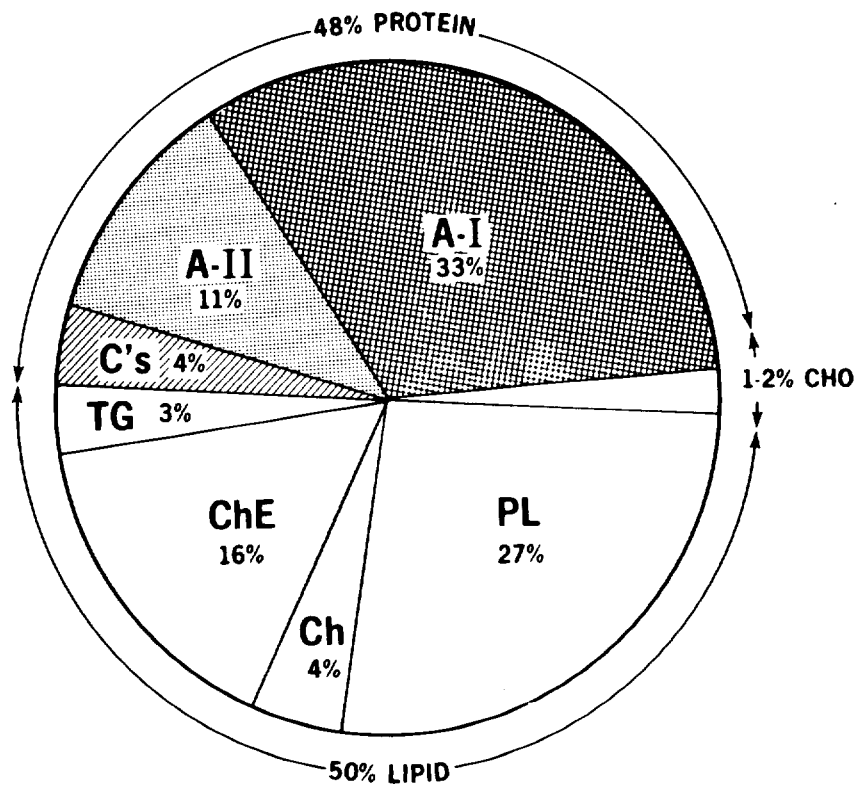


FIG. 3. Representation of the «average» composition of plasma HDL. Abbreviations: TG, triglyceride; ChE, cholesteryl esters; Ch, cholesterol; PL, phospholipids; C's, apoproteins C-I, C-II and C-III; A-I, apoprotein A-I; A-II, apoprotein A-II; amounts are percentage mass of dehydrated lipoproteins isolated between D 1.063 to 1.21.

APOPROTEINS - 1960

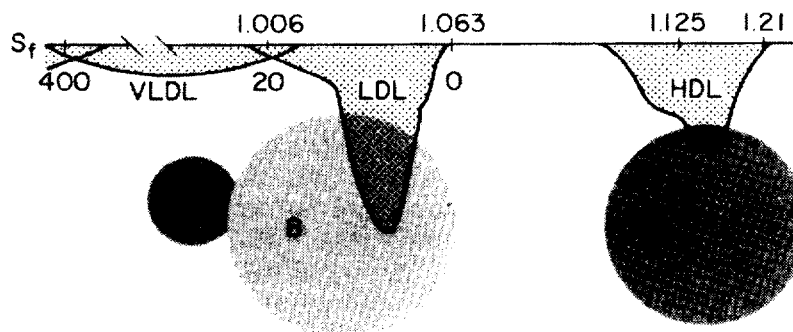


FIG. 4.

available. Others, particularly the Shores in California (18) had demonstrated by then that there might be more than one HDL protein, and Alaupovic and co-workers in Oklahoma (19, 20) found evidence that VLDL contained a «C-protein» different from the major proteins in LDL and HDL.

THE C-PROTEINS

Although Tangier disease was not forgotten, we soon became more preoccupied with the proteins of triglyceride-rich lipoproteins. Using a new classification scheme for patients with hyperlipoproteinemia (21), we and others were turning up increasing evidence for heterogeneous genetic defects associated with hyperglyceridemia. To those interested in metabolic defects the VLDL and chylomicrons were systems compelling much study, for their functions spell greater immediate consequence in maintenance of caloric economy than is apparent in the more sedentary concentrations of LDL and HDL.

VLDL are triglyceride-rich particles, having a diameter of 300 to 1000 Å, that first become visible in the Golgi bodies of the liver (22) and small intestine. They remove glycerides from the liver that accumulate as the result of net synthesis controlled by a number of operators. One is the metabolism of free fatty acids (FFA), another that of glucose. In the small intestine, VLDL are elaborated along with the larger (1000 to 5000 Å) chylomicrons that bear dietary glycerides into the circulation.

Between 1966 and 1970, particularly through the work of Dr. Virgil Brown, we succeeded in isolating four apoproteins from delipidated plasma VLDL. One of these was the large and already known apoprotein B, the protein of LDL, which has yet to be characterized. The other three were new ones, and had much lower molecular weights than apoB, between 6000 and 9000 daltons (23-25). We first designated them according to their carboxyl-terminal amino acid residues (23), but it has become desirable to use a single nomenclatural system for apolipoproteins, and the simplest proposed thus far is the ABC nomenclature of Alaupovic and colleagues (26, 27), which I will use here. I have discussed elsewhere the pros and cons of the several nomenclatural systems (28).

ApoC-I. The first protein separated from the C-protein group by DEAE chromatography is C-I. The complete sequence of this 59 amino acid protein (Fig. 5) was determined in Bethesda last year (29), and recently confirmed in Houston (30, 31). This protein is an avid binder of lipid, an event which greatly increases its content of α -helix. I will return later to this general phenomenon.

ApoC-II. The amino acid sequence of the second protein which we isolated has not yet been determined. C-II has a molecular weight of about 9000 daltons. It is of great interest because of the discovery (32, 33) that it is an «activator» of the enzyme lipoprotein lipase. This enzyme is located in the endothelium of capillaries and larger vessels, the concentration being especially high in adipose tissue and lacteal glands. Lipoprotein lipase promotes hydrolysis of chylomicrons and larger VLDL particles and breaks them down to smaller remnants, some of which remain longer in plasma as LDL.

ApoC-III. Brown *et al.* (23) and the Shores (34) independently isolated the most abundant of the C-proteins, C-III. We found it to exist in several forms, a polymorphism shown to be related to varying content of sialic acid (23). ApoC-III, like apoB is a glycoprotein, in contrast to the other apoproteins thus far characterized. The amino acid sequence of C-III (Fig. 5) was determined in our laboratory in 1972, and was the first of the apoproteins to be so characterized (35, 37).

between HDL and chylomicrons and VLDL as these triglyceride carriers emerge from the liver and intestinal cells (39). As the triglyceride is stripped away during catabolism of these particles to LDL, some of the C-proteins return to HDL. We have presented indirect evidence, based on studies of abetalipoproteinemia (40, 41), and Windmueller and others have developed other information (42) which suggests that the C-proteins are not essential for secretion of triglycerides from cells. Rather, apoB, and possibly certain other still poorly defined apoproteins, are required. The impression is, today, that the welcoming embrace of C-proteins accorded chylomicrons and VLDL as they enter plasma is nevertheless of considerable importance in the stabilization and further metabolism of these particles, including their availability as «supersubstrates» for lipase action. One would expect then that deficiency of C-proteins might have disruptive influence on plasma triglyceride disposal. And we shall presently see possible signs of this when we return to Tangier disease.

HDL APOPROTEINS

As revelations concerning the C-proteins were appearing, the HDL proteins had become the subject of similar dissection in a number of laboratories. Despite some evidence of heterogeneity based on immunochemical studies and partial fractionation, up until 1968 it was generally believed that apoHDL (or apoA) was a single subunit having a molecular weight from 21,000 to 31,000. In that year the Shores published first the partial characterization of two principal components in the HDL proteins (43, 44). This was fairly promptly confirmed by others (45-47). The Shores originally reported that one of their two proteins contained carboxylterminal threonine. The «apo-thr» in the literature, however, must now read apo-Gln-I (in the carboxyl-terminal nomenclature) or simply apoA-I; the original analyses have proved to be in error (27). The other major HDL apoprotein also has carboxyl-terminal glutamine and is called apoA-II (Fig. 3).

Many methods are used to isolate apoproteins in HDL; the most commonly used combine gel filtration and ion-exchange chromatography to achieve maximum purification (Fig. 7). The yield of proteins from delipidated normal human HDL is as follows: the greatest mass is apoA-I (65-75 percent); A-II represents 20-25 percent, and the rest consists of the C-proteins and very small amounts of an arginine-rich protein and others that have not yet been characterized (18, 34, 48).

ApoA-II. Brewer and Lux and several other co-workers in our group completed the determination of the primary sequence (Fig. 5) of the smaller of the two major HDL proteins last year (49, 50). The structure of A-II is unusual, perhaps unique among human proteins, in that in man it appears to consist of two identical monomers, each 8690 daltons in molecular weight, joined by a single sulfhydryl bridge at position 6 (49). The amino termini are «blocked», consisting of pyroglutamic acid. Each monomer contains a single half-cystine. There is no histidine, arginine or tryptophan, and no carbohydrate. In the circulating HDL in plasma of most animals, A-II is apparently present as the monomer, and the relative amounts of A-II in man and possibly some other primates are greater than they are in other species.

Genetic relationships. From the primary structures of the several of the apoproteins I have just described it is possible to make comparisons that shed light on their genetic relationships. All three apoproteins have their homologues in species that developed long before man (28). The alignment of the amino acids in C-I, and the first 59 residues in C-III and A-II is so similar as to suggest that all three have arisen from a common ancestral gene (Table 4) (51, 52). It is noteworthy that the phospholipid-binding portions of these proteins

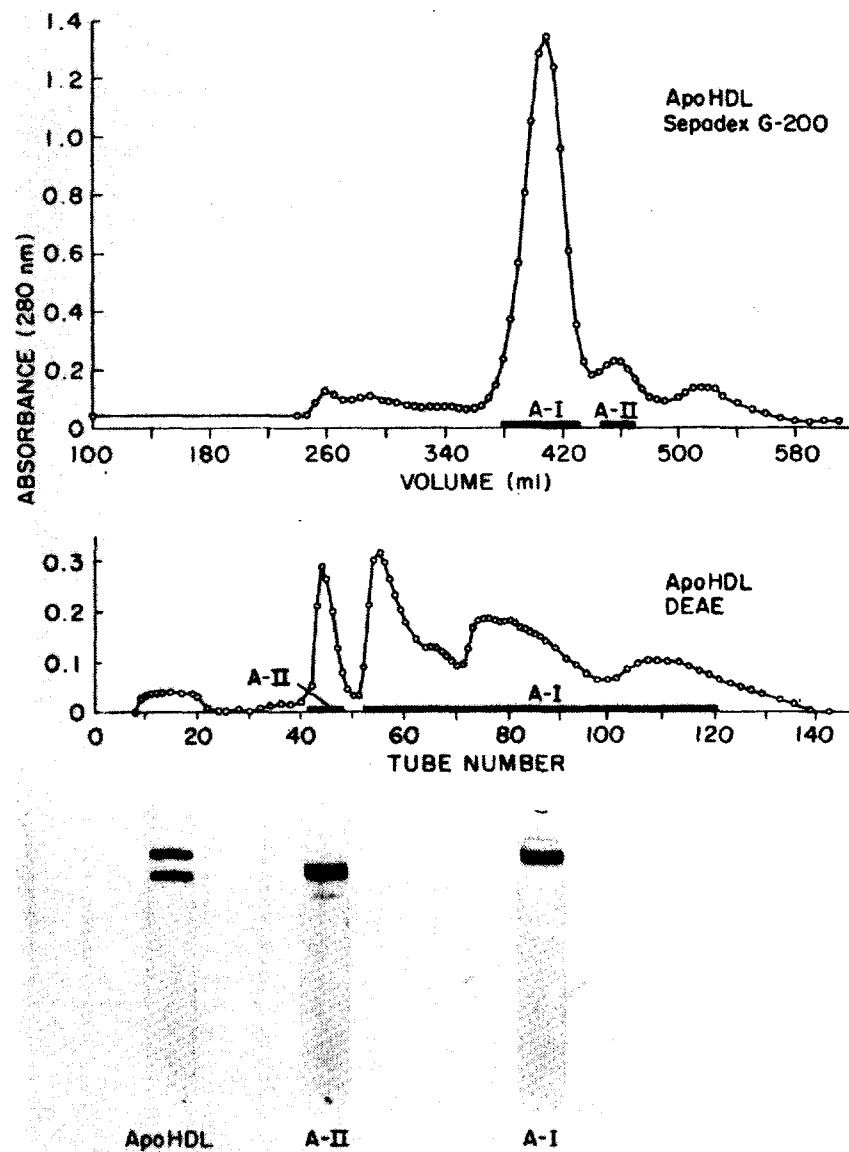


Fig. 7. Separation of delipidated HDL apoproteins by gel filtration (top), or by DEAE ion exchange chromatography (middle). The multiple peaks containing A-I on DEAE all migrate to the same position after polyacrylamide gel electrophoresis (below). The peak following A-II on Sephadex contains the C-proteins.

appear to be located in the carboxyl-terminal, rather than the very similar amino-terminal ends (53-55). It is intriguing to conjecture what properties other than lipid-binding these proteins shared prior to their divergent evolution.

ApoA-I. The other major apoprotein in HDL is A-I. In the adult human male it is second to apoB in plasma concentration among all the apolipoproteins. In some human females and children, and in most other species, it is the dominant apolipoprotein in plasma. The 245

TABLE 4.—Genetic relationships between apoproteins

		Alignment Score	P
C-I	C-III (1-59)	4.56	10^{-5}
C-I	A-II (1-59)	4.56	10^{-5}

Alignment scores were obtained by Dayhoff and Barker for comparison of the complete sequence of C-I and shortened sequences of C-III and A-II. The p value is the calculated probability that the similarities in amino acid sequence would occur by chance (51, 52).

amino acid chain of A-I has just been sequenced by Jackson and co-workers in Houston (56). This is a larger protein than A-II, having a molecular weight of approximately 28,000. It contains no cysteine or cystine or sugar residues. The recombination *in vitro* of lipids with A-I, or with some of its peptide segments, have also been extensively studied. It is apparent that not only the primary structure of apoproteins, but also their conformation is ultimately related to lipid binding and the formation of lipoproteins.

MODELS OF HDL

The quaternary structure of lipoproteins is still poorly understood. Available models have none of the three-dimensional elegance or accuracy possible for hemoglobin or the cytochromes. Yet certain progress has already been made toward construction of crude models of HDL. This has been made possible by the availability of pure apoproteins, especially A-II and A-I, which can be completely delipidated, yet disaggregated so that they may be dissolved in aqueous buffers and presented to lipids in recombination experiments.

In plasma lipoproteins, there are no covalent bonds between the lipids and apoproteins. The forces that hold them together may be electrostatic, as between the charged polar head groups of the phospholipids, principally phosphatidyl choline and sphingomyelin, and free carboxylic or amino groups on certain amino acids. Or they may be held together by hydrophobic bonds, which depend upon interactions between non-polar amino acids and mainly the long alkyl chains of fatty acids in the phospholipids, steryl esters or triglycerides. Inspection of the primary sequences of the apoproteins does not reveal long segments of non-polar amino acids or necessarily an unusual number of adjacent pairs of negative and positively charged amino acids (28, 56), and thus does not immediately reveal the nature of the forces holding lipoproteins together.

Most of the attempts to reconstitute lipoproteins have thus far been partial ones, consisting of the recombination of apoproteins with either phosphatidyl choline or sphingomyelin. When this is undertaken it is observable by appropriate methods that the apoproteins show an appreciable increase in α -helical structure. This leads to the conclusion that changes in the conformation of the apoprotein chain, or portions of the chain, in the presence of phospholipids, induces «amphipathic properties» (57-61); i.e., the helix brings the amino acids into an alignment that promotes either electrostatic or hydrophobic bonding with the lipid.

A number of ingenious approaches to this end have been used. Deserving of particular mention at this time is the work of Dr. Gerd Assmann, who came from Cologne to work with me several years ago. Recently he has carried out independently a study of lipid-protein binding by the A proteins that takes advantage of excellent nuclear magnetic resonance (nmr) equipment in Bethesda.

From his experiments it appears, first of all, that HDL «particles» likely represent micelles (Fig. 8) (57-60), as early x-ray diffraction patterns had suggested (62, 63). Thus, the polar head groups of the phospholipids are all oriented on the outer surface of a spherical micelle. This is determined by noting that all of the phospholipid in HDL is available for relation with Eu^{++} a heavy ion that presumably cannot penetrate to the center of the micelle (57).

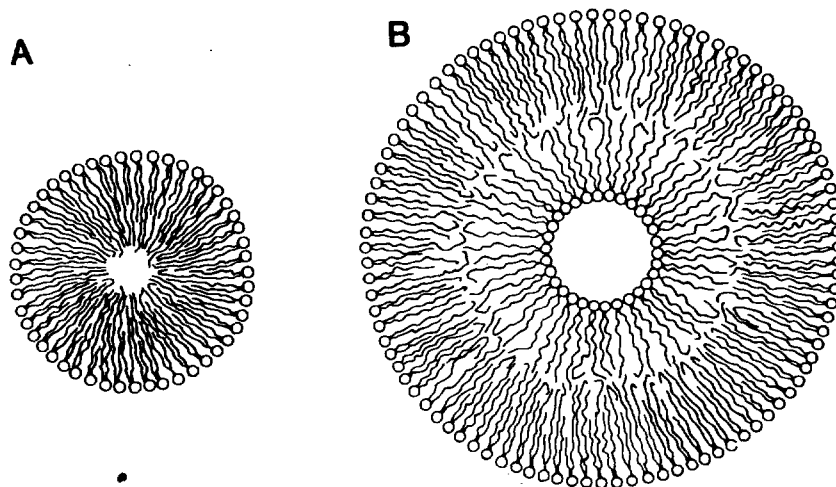


FIG. 8. Two possible basic models of arrangement of phospholipids in plasma lipoproteins. In A, all the polar head groups are oriented to the surface or water phase as a micelle. In B, a bilayer exists with polar head groups outside and within. Data suggest HDL is a micelle and LDL may be a bilayer.

One is inclined to believe naturally that the proteins, with their hydrophilic properties, will be located on the surface of the micelle. To ascertain if this is so, and to determine the nature of lipid protein interactions, phospholipids have been synthesized which are enriched with the natural isotope ^{13}C in a carbon atom located in either the polarhead group or in one of the fatty acid chains. The nmr pattern of ^{13}C allows determination of how constrained or tightly bound such an atom is to adjacent molecules. A corollary approach is to use phospholipids labeled with the radioactive atom ^{14}C so that quantitative measurements of the degree of binding of phospholipids to the several apoproteins can be made.

To summarize many experiments in the briefest terms, the data collected by Assmann et al. suggest the following. ApoA-II seems to have a higher affinity for phospholipid than does ApoA-I (57-60). When A-II is recombined with ^{13}C -phospholipids, the nmr patterns indicate greater constraint of the carbons in the alkyl fatty chain than of those in the polar-head groups of the phospholipids. This suggests that the binding of apoprotein to the fatty acid portion of the phospholipid by hydrophobic forces is more important than the electrostatic binding of the apoprotein (A-II) to polar head groups of the phospholipids. The data do not

absolutely prove this point because controls are still required to show that the constraint due to electrostatic bonding of a phospholipid head group is always detectable by nmr.

It is possible to construct three-dimensional models of A-II in helical form (56, 59, 61). When this is done, it becomes apparent that a region containing non-polar amino acids is formed on one surface of the helix. A continuous non-polar surface extending about the same distance as the length of the alkyl chain of an 18 to 20 carbon fatty acid is possible (59). The evidence for hydrophobic bonding may be interpreted to imply that a significant portion of apoprotein A-II is oriented parallel to the fatty acids and thus is embedded for an appreciable distance into the lipid core of the lipoprotein.

The weaker binding of A-I to phospholipids, according to Assmann's data, suggests that in the formation of HDL, A-II may be the principal lipid-binder and that A-I could be attached to the resultant particle primarily by protein-protein interactions. This is consonant with prior evidence that A-I is less closely bound to the mass of HDL lipid, A-I-phospholipid particles being rather easily dissociated from HDL by high centrifugal fields, high salt concentrations, freeze-thawing and other physical changes (64, 65).

Other workers in Houston, including former colleagues in Bethesda, Jackson and Gotto, have adopted a different point of view, mainly on theoretical grounds (61). They, too, have constructed helical models of apoproteins C-I and A-I (30, 56) and observe that the side opposite the non-polar surface contains pairs of oppositely-charged amino acids. The amino acids containing a free base, such as lysine and arginine, are always located laterally, while the free carboxyl groups of glutamic and aspartic acids are located mesially, along the axis of the helix. This latter arrangement of acid-base pairs seems to be unique for plasma apolipoproteins among all known proteins (30). Thus, these workers argue that electrostatic binding of phospholipids to apoproteins theoretically is at least as important as hydrophobic bonding. In their view, the proteins in lipoproteins are probably oriented perpendicular to the surface of the lipid micelles.

Thus, as we now turn back to Tangier disease, keep in mind the distribution of apoproteins in the normal (Fig. 6) as well as the following points: (1) normally, human HDL contains two major apoproteins A-I and A-II; these are present in a fairly constant ratio by mass; there is also present a smaller but physiologically important amount of C-proteins. (2) It is not known whether any of these apoproteins can form independent lipoprotein particles or not, and the interrelationships between A-I and A-II in maintaining HDL concentrations in man are not understood; and (3) a suggestion has been raised, from some *in vitro* experiments, that A-II might be the principle lipid-binder in HDL and thus form a primary particle to which A-I may be bound.

TANGIER DISEASE, 1974

Since the discovery of the peculiar tonsils and HDL «deficiency» on Tangier Island events have broadened the attention focussed on this disease to the world beyond the island. Intermittently, Tangier disease has been the subject of intensive laboratory investigations, particularly some quite recent ones and exciting ones in Bethesda. By now 15 other patients with an apparently identical abnormality have been found, none of these new cases being from Tangier Island. Eight others are American, two are German, two Australian, one Swiss, and one from New Zealand (1-8, 66-68). Familial involvement has been confirmed in 3 more kindreds and the cardinal clinical signs reinforced. As other patients have been discovered—and as the original patients have matured—several new features have been

added. One is the presence in all affected adults of deposition of lipid in the cornea, visible evidence of an inexorable increase in cholesteryl ester accumulation with time, even in avascular tissues. The other is more alarming; it is the appearance in the majority of patients of recurrent polyneuropathy, which has interfered with both motion of the limbs and of the eyes, and left some older patients without protective sense of pain (69, 70).

NEW BIOCHEMICAL DATA

I will have more to say about these clinical findings later, but more immediately pertinent are the results of applying to Tangier disease some of the newer knowledge about lipoproteins I have just described. Being one of nature's own experiments, the disease offers some almost unique opportunities to test and to shape some of the thinking about lipoprotein structure and function.

Tangier HDL. As I have indicated, from the beginning we were aware that the first patients with Tangier diseases had very small amounts of protein in plasma that reacted to antisera made against HDL (1). Later, we found this material sometimes had unusual migration on immunoelectrophoresis and we called it HDL_T (5, 6). We even had the rare opportunity to observe the disappearance from the plasma of normal HDL transfused into one of our patients with Tangier disease who had to undergo open heart surgery. What we could not know at that time was that the antisera that saw «HDL_T» were polyvalent; i.e., reactive against more than one HDL apoprotein and in different titer.

The application to Tangier plasma of techniques for isolating pure apoproteins is very difficult. Several pints of plasma have to be removed from any one patient by plasmapheresis in order to obtain enough HDL for any reasonable analyses. In the first chemical analyses of the density 1.063-1.21 (HDL) fraction of Tangier plasma (71), unequivocal evidence was obtained that some A-II was present, immunochemically identical to normal A-II and co-migrating with the normal A-II dimer (Fig. 9). Moreover, this A-II was mainly confined to

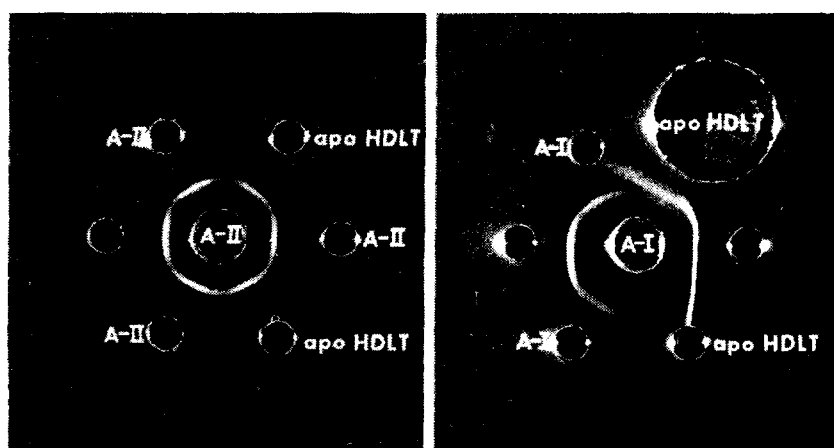


Fig. 9. Immunochemical analyses showing presence of apoA-II in Tangier plasma (HDL-T) (left panel); in the right panel, evidence is shown that a very small amount of apoA-I is present. The HDL-T well contains 15 times the amount of material as in the similarly located well in the left panel. The right-hand (unlabeled) well in the right panel contains anti-albumin serum. This shows albumin contamination in the HDL-T preparation used. Data adapted from (71).

the HDL density region, there being only traces or none in other lipoproteins or in the density > 1.21 infranatant. A very small amount of immunochemically reactive A-I was also found in the HDL density range (71) (Fig. 9).

From recent unpublished experiments, performed with Drs. Assmann, Peter Herbert, Trudy Forte, and Mr. Robert Heinen, we now know a good deal more about HDL_T. It appears that the A-I in Tangier plasma is confined almost exclusively to the «very high density» protein fraction of density > 1.21 (72). The amounts present there are perhaps about half of the A-I normally found in this plasma fraction. Because this A-I floats in the ultracentrifuge at density of 1.25 we assume it is associated with some phospholipid (lysolecithin) as is the case with the A-I in the «very high density» region in the normal state.

THE A-II PARTICLE

The extremely small concentration of lipoprotein material in the HDL density range in Tangier plasma nevertheless is extremely interesting. The highly concentrated «HDL fraction» (isolated at density 1.063 - 1.21 and washed once to remove albumin contamination) from an adult with Tangier disease has been photographed for us under the electron microscope by Dr. Forte of the Donner Laboratory in Berkeley, who probably has more experience than anyone else in the world with such examination of lipoproteins. Instead of the fairly uniform field of particles of 100-150 Å in diameter obtained with normal HDL (Fig. 10), the concentrated Tangier «HDL» contains clumps of larger particles, from 200 to over 1000 Å in diameter, against a field of very small particles (72).

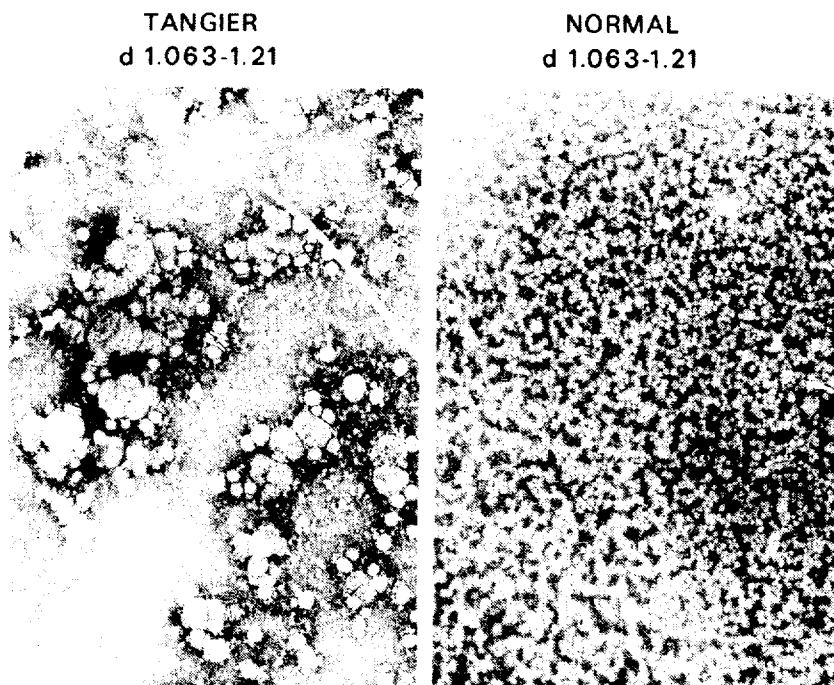


FIG. 10. Electron photomicrograph showing concentrated Tangier «HDL» (the fraction isolated between densities 1.063-1.21) (left) and HDL from a normal subject (right). The solid line represents 1000 Å (photomicrograph prepared by Dr. G. M. Forte).

When this Tangier HDL is chromatographed on 8 percent Sepharose, it is separated according to size into two populations (72). The largest share emerges in the void volume and contains the very large particles of irregular size. These consist of apoB, triglyceride, phospholipids and cholesterol. The content of sterol seems to be greater than the content of phospholipids, as is the case in LDL. This material conceivably is the polymorphic LDL known as LpA (73) or «sinking pre-beta» (74) and thus is a usual constituent of HDL that is normally obscured by the mass of HDL particles. Alternatively, these particles may be the residue from abnormal catabolism of triglyceride-bearing lipoproteins in Tangier disease (75). There is a suggestion that they may principally arise from chylomicrons for they are present in greatest amounts after high-fat feeding and may not be visible when fat has been removed from the diet for several days (75).

Included in the column and eluted later is a small fraction of the total material present in a concentration of perhaps a milligram per liter of plasma. These are lipoprotein particles that contain exclusively apoprotein A-II; no apoA-I is detectable immunochemically or by fluorescence in the tryptophane region (A-I, unlike A-II, contains tryptophane). In addition to the A-II protein, these particles contain lecithin, sphingomyelin, cholesterol, cholesteryl esters and possibly small amounts of triglycerides. As determined thus far by qualitative thin-layer chromatography, these lipids appear to be present in the same proportions as in native HDL.

The «A-II particle» is shown in the electron photomicrograph in figure 11. Its particle size distribution peaks at 60 Å and there is no overlap with the diameter of the larger normal HDL₂ particles. The normal particles isolated in the HDL₃ region (density 1.12 - 1.21), which normally are much richer in A-I than A-II, are intermediate, and overlap somewhat in size with both the HDL₂ and the Tangier «A-II» particles (72).

TANGIER A-II

Trypsin hydrolysates of lipid-free Tangier and normal A-II have very similar characteristics, although a possible significant difference cannot be excluded without sequence analyses. Purified Tangier A-II recombines with sphingomyelin *in vitro* in a fashion apparently identical to those worked out for normal A-II by Assmann (72). The appearance of the resultant recombinant disks is the same (Fig. 12). Moreover, the trypsin hydrolysates of the normal and Tangier recombinant particles (the phospholipid being present during hydrolysis) are indistinguishable. This is noteworthy because when the combination of phospholipid with human A-II is attacked by trypsin, unusual fragments result which suggest that one of the monomeric arms of A-II is differently bound to phospholipid than the other. Perhaps more importantly, the hydrolysis products ascribable to A-II that are obtained from whole normal HDL are also identical to those obtained from the «A-II particle» in Tangier disease. Lipid binding to A-II in the latter particles, therefore, would appear to be comparable with that in normal HDL.

FURTHER MEANING OF THE «A-II PARTICLE»

These very recent and preliminary studies suggest that the Tangier «A-II particle» may be a copy of the lipid A-II complex normally present in HDL. At the least, the Tangier «A-II particle» has given us the first glimpse of a circulating lipoprotein in plasma consisting only

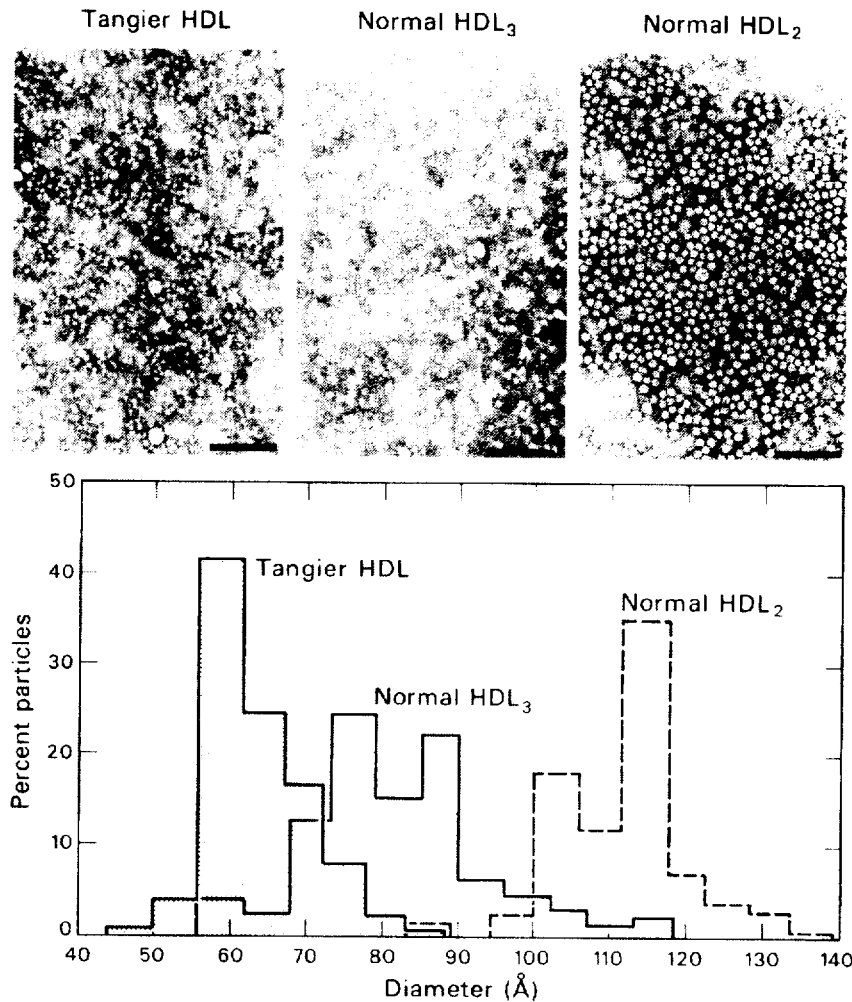


Fig. 11. Electron photomicrographs of the Tangier «A-II particle» (left panel), normal HDL₃ (middle) and normal HDL₂ (right panel), as well as particle size range (below). The solid line represents 100 Å (photomicrograph and particle size analyses courtesy of Drs. G. M. Forte and Alexander Nichols).

of A-II. This is the kind of revelation one can sometimes obtain from studying the result of a mutation.

We also must be allowed some further speculation about normal HDL formation and the specific gene locus in Tangier disease. As I indicated earlier, we have not yet had time to prove that Tangier A-II is completely normal in either molecular structure or function, but it is likely that this is so.

If Tangier A-II is the same as the normal gene product, then what we have exposed in Tangier plasma may be the primitive essential core of normal HDL. We will assume for purposes of speculation that this «core» takes the appearance of a previously drawn model (60) in which A-II is partially embedded in the lipid core of a phospholipid-cholesterol micelle (Fig. 13). This particle of 60 Å diameter is half to two-thirds the size of the normal HDL₂ particles. Arguments can then be advanced that, in the complete HDL, the central

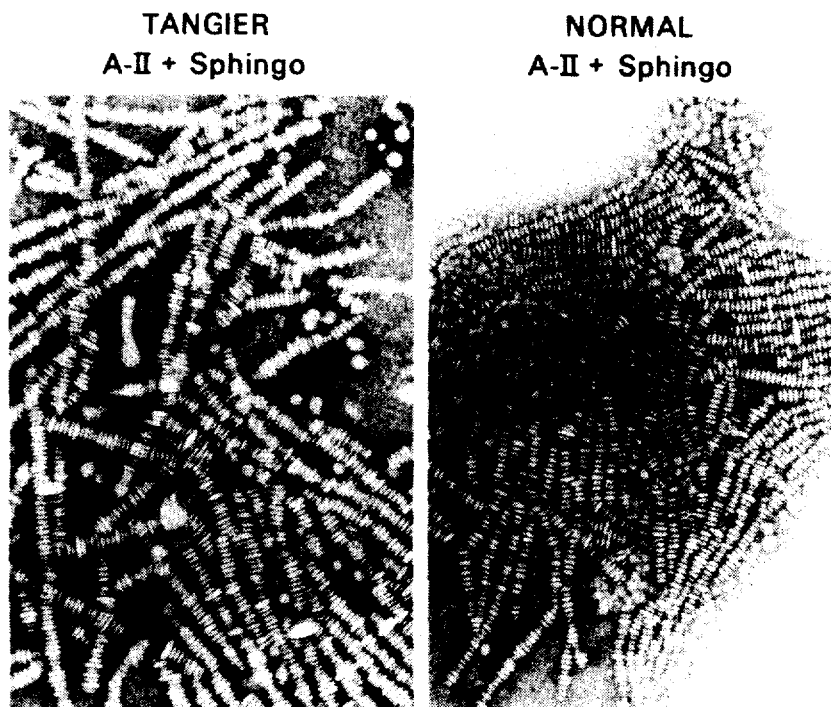


FIG. 12. Electron photomicrographs of recombinant particles achieved by mixing Tangier A-II (left) and normal A-II (right) with sonified liposomes of sphingomyelin («Sphingo»), followed by isolation by ultracentrifugation and column chromatography (57, 58). Solid line represents 1000 Å (photomicrographs prepared by Dr. G. M. Forte).

particle is partially surrounded by apoA-I, which is held there by either protein-protein (60) or protein-phospholipid (61) interactions, or both (Fig. 14). To obtain the size of the normal HDL₂ particle, it may also be that the amount of micellar lipid in the «A-II particle» is also somewhat expanded.

It seems to me that the most *likely* defect in Tangier disease is a structural mutation in apoprotein A-I which prevents its proper binding either to lipid or to protein. We have begun to isolate small quantities of fairly pure A-I from Tangier plasma. If it proves to be a mutant protein, it could provide assistance in settling the uncertainty of which of the conformational amphipathic properties of A-I—and other apoproteins—is the more critical provider of the forces that hold lipoproteins together.

There are other possible loci of the Tangier mutation: a subtle defect in A-II structure, regulatory defects limiting the production of A-I or A-II or both, or some other more obscure possibility affecting lipoprotein assembly or catabolism.

Regardless of the locus involved, the evidence in Tangier disease strongly suggests that survival of the usual HDL particles in plasma is dependent upon interactions between the two apoproteins A-I and A-II. Lipoproteins formed by combination of lipids with either one or the other alone do not persist in any appreciable concentrations.

A schematic summation of the lipoprotein and apoprotein concentrations in Tangier plasma is shown in figure 15. From Tangier disease we have also learned something about the C-apoproteins (also depicted in Fig. 15) and their relationships to HDL particles.

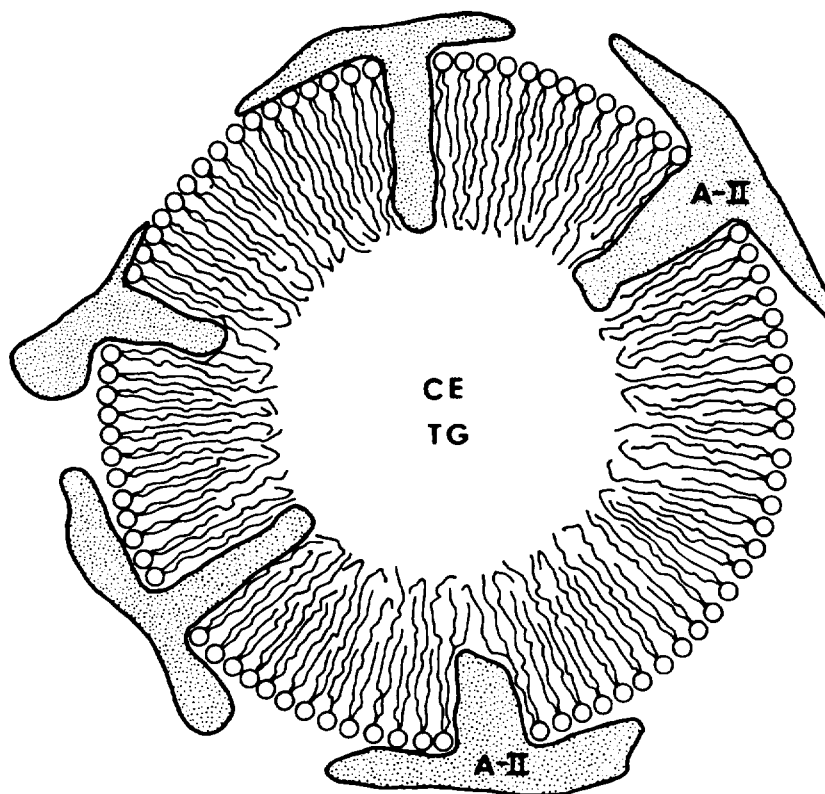


Fig. 13. A model illustrating possible interactions between apoA-II and phospholipid micelle in HDL (after reference 60).

C PROTEINS

In Tangier plasma, it can be seen that the complexes normally created by apoproteins A-I and A-II, and their lipid complement, also are required for retention of the C-proteins in the HDL region. All of the established C-apoproteins are present in Tangier VLDL, but only traces are present in the HDL density region; and these are probably found in the large «residual» particles that contain no A apoproteins. The net effect in Tangier plasma is a severe reduction of the total C-protein concentration in plasma, perhaps to less than half of normal (76).

What are the consequences of this reduction in C-proteins or of the ability to recycle these proteins between HDL and chylomicrons or VLDL? As I have remarked earlier, secretion of exogenous or endogenously synthesized glycerides does not seem to be impaired. If the C-proteins are not critical for such excretion (28), we would not expect evidence of malabsorption or triglyceride accumulation such as occurs in the converse disease, abetalipoproteinemia (6). Yet, there are subtle signs in plasma of defective triglyceride catabolism or removal in Tangier disease, such as protracted fat tolerance curves, abnormal composition of chylomicrons (5), and generally elevated plasma triglyceride concentrations.

If chylomicrons or perverse remnants of them linger too long in plasma, there is also evidence that the usual pathways for removal of chylomicrons and VLDL are short-circuited.

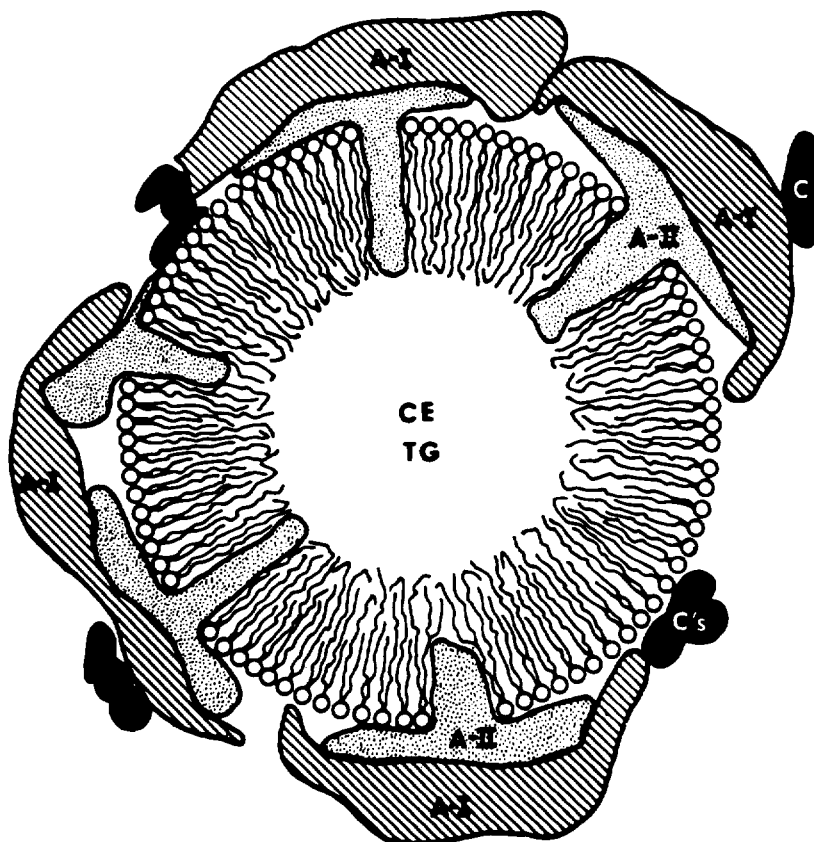


FIG. 14. Model illustrating possible addition of apoA-I and the C-apoproteins to the A-II particle core shown in Fig. 13.

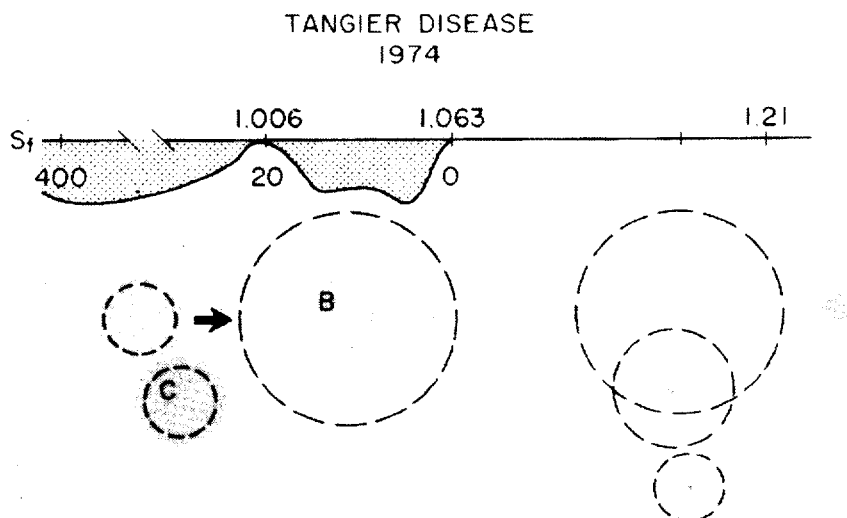


FIG. 15. A schematic representation of lipoprotein and apolipoprotein location and concentrations in Tangier plasma. See Fig. 6 for comparison with the normal state.

The concentration of their usual end products, LDL, is quite diminished and the composition of the remaining LDL is not normal (Table 5) (75).

Two enzymes important in the normal catabolism of triglyceride-bearing lipoproteins are lipoprotein lipase and lecithin:cholesteryl acyl transferase (LCAT). The first catalyzes the hydrolysis of triglycerides. It is displaced briefly in plasma after heparin and determination of plasma post-heparin activity is as much a measurement of the ability of heparin to displace the enzyme as it is of the activity in tissues. In Tangier disease, post-heparin lipoprotein

TABLE 5.—LDL Composition

	FC	CE	TG	PL	Prot.
Tangier	5	10	40	20	25
Control	10	35	10	20	25

(gm/100 gm dry wgt)

Abbreviations: FC, free cholesterol; CE, cholesteryl esters; TG, triglycerides; PL, phospholipids; and Prot., protein (75).

lipase activity is sometimes normal (68) but in one Australian patient (66) and in several of ours we have found such activity to be significantly reduced. Could this be due to relative deficiency of the C-protein activator of the enzyme? LCAT catalyzes esterification of cholesterol in plasma and may help stabilize chylomicrons and VLDL as they are being stripped of triglycerides (77). LCAT is believed to be activated by apoA-I (78) and also is thought to depend upon HDL phosphatidyl choline for the fatty acid it must transfer to cholesterol. LCAT activity is present in Tangier plasma and appears to be normal by *in vitro* measurements (68). It cannot be excluded that *in vivo* the activity of this enzyme may not be normal.

QUESTIONS OF HDL FUNCTION

The consequences of failure to maintain normal HDL in plasma, therefore, are seen in the structure and metabolism of other lipoproteins and suggests that HDL is importantly involved in the processes of triglyceride transport and metabolism. In assessing whether this is probably the only role of HDL, I should like to end by returning to the tissue changes in Tangier disease. I have the impression that if we could decipher the maps provided by the morphologists, particularly the ultrastructural photographs made through the electron microscope, we can understand more of the physiological functions that have evolved for the lipid-protein complexes we know as HDL.

The predominant change in the Tangier tissues is the conversion of large numbers of histiocytic cells in many organs to depots of cholesteryl esters. The sterol is stored within the cytoplasm in droplets or crystals unbound by membranes (Fig. 16) (8). Of all the tissues examined none has an appearance more striking than that of the Schwann cells in small nerves, whether seen in the skin (Fig. 17) or elsewhere (8, 79). These cells, which wrap

around the axons are filled with lipid droplets in Tangier disease. It seems that they may be burdened with unwanted lipid and compromise the nerves they surround in a peculiar remitting fashion in most of the patients with this disease. One cannot help but remember that the Schwann cells are the manufacturers of the myelin sheath, which is also a lipoprotein. It is unlikely, but not impossible, that myelin and HDL have some arcane relationship as lipoproteins that underlies involvement of the Schwann cells in the storage process.

A minority of foam cells visible in the bone marrow of patients with Tangier disease deserve special mention (Fig. 18) (8). Unlike most of the foamy histiocytes, these cells contain vesicles that are bound by lysosomal membranes and have the appearance of material that is drawn into the cells by phagocytosis or pinocytosis. Thus there is evidence

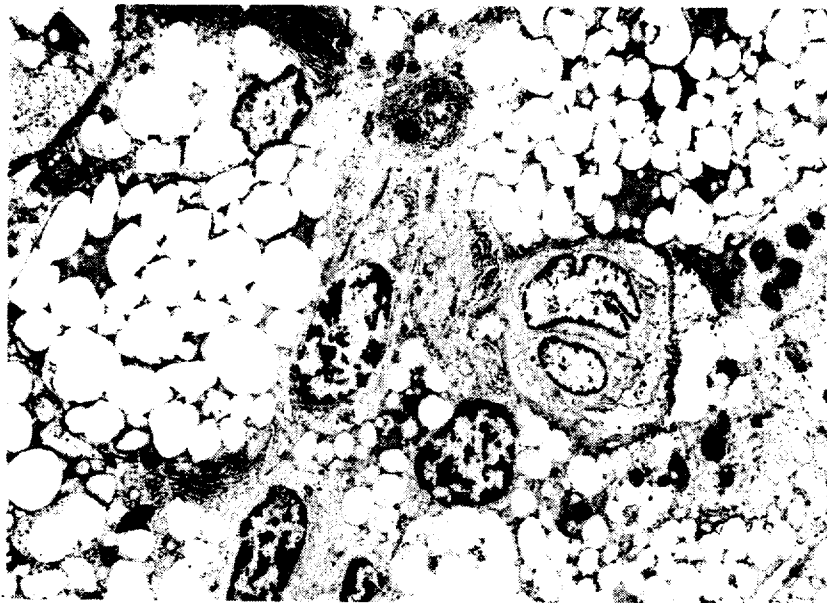


Fig. 16. Electron micrograph of section of tonsil from patient with Tangier disease. Droplets or crystals of lipid are contained in the cytoplasm unbound by limiting membranes. Previously fixed with phosphate-buffered formalin, the tissue was refixed with 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2. After washing with several changes of 5% sucrose in 0.1 M phosphate buffer, pH 7.2, the tissue was post-fixed with cold 1% OsO_4 in Millonig's phosphate buffer, dehydrated and embedded in Maraglas. Ultrathin sections were stained with uranyl acetate and lead citrate X 5100 (courtesy of Dr. Victor Ferrans).

that some circulating lipid particles —perhaps the remnants of abnormal chylomicrons or VLDL that I referred to earlier, or perhaps bits of HDL discarded because of their instability in plasma— are being handled as foreign bodies. I believe it is very likely that absence of HDL does cause triglyceride-rich particles to be removed by scavenger mechanisms. For this to result in some of the changes in tissue lipid composition observed, we must assume that all lipids other than cholesterol are handily disposed of, that the sterol remaining becomes largely esterified, and that the intracytoplasmic membranes around the deposits eventually disappear.

However, I think it is possible that lipid also comes to be stored because another, more universal role of HDL may go untended in Tangier tissues. This would have to be a function concerned with regular removal of cholesterol from cells in such a way that reasonable intracellular concentrations are maintained. All cells synthesize cholesterol; only the liver is able to degrade the side chains to an excretable product, bile acids. Thus, some device to sweep excess sterol to the liver is plausible. Again the storage of esters requires that sterol be esterified as it begins to pool in the cell and that there is a functional inadequacy of cholesteryl ester hydrolase activity to handle the resulting accumulation.

Mysteries remain to be solved, both about Tangier disease and about structure and function of plasma lipoproteins. No doubt other diseases that result from mutations at different loci will be discovered, and perhaps they will provide some of the additional needed illumination.

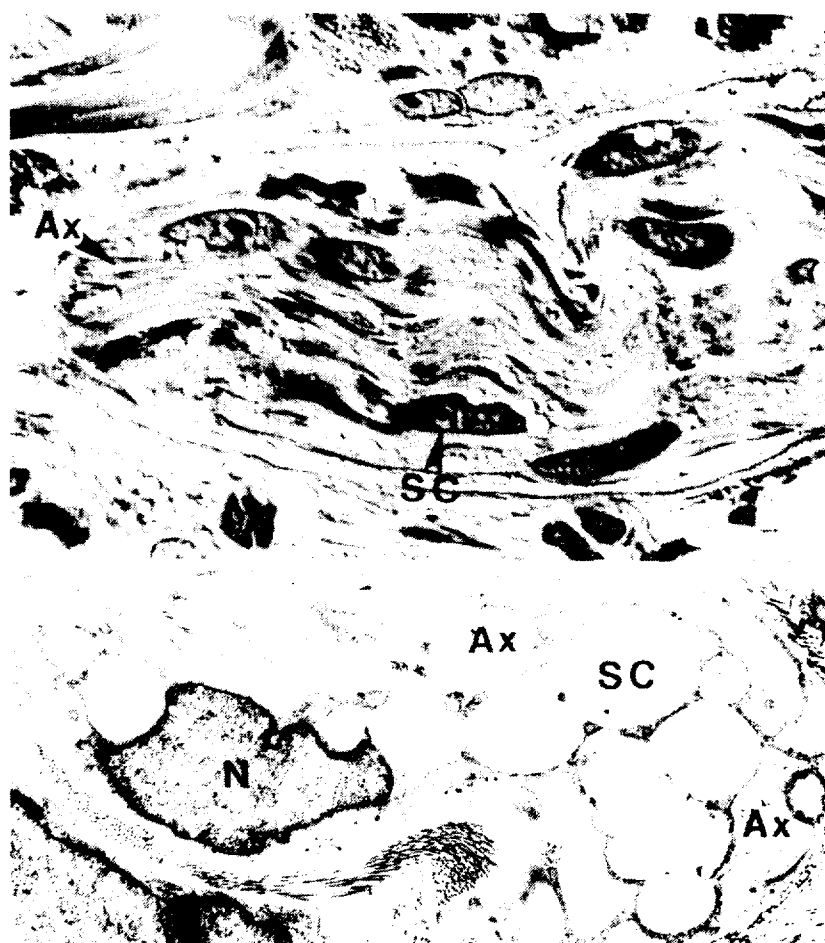


FIG. 17. Photomicrographs of biopsy materials from patient with Tangier disease, including a nerve. Upper view is skin, taken using Nomarski interference contrast optics, X 1000. Lower view is an electron micrograph of jejunal submucosa showing a Schwann cell (SC) X 8000. The latter contains a nucleus labeled (N) and envelopes several axons (Ax) (preparation as in Fig. 16, by Dr. Victor Ferrans).

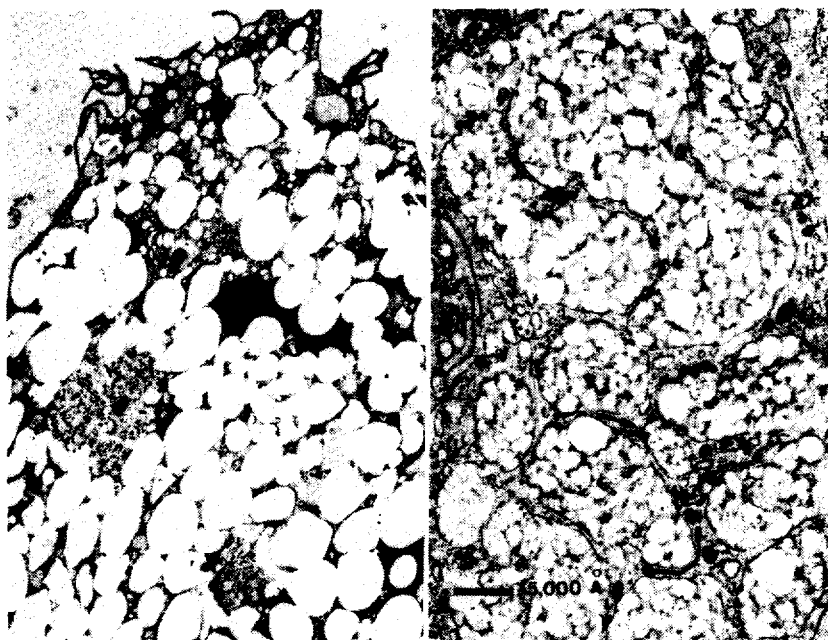


FIG. 18. Bone marrow foam cells in patient with Tangier disease. Left, predominant type of vacuolated cell in which lipid is present without limiting membranes, X 6000. Right, a minority of cells contain large, irregularly-shaped lipid-filled vacuoles which are bound by trilaminar membranes, suggesting uptake by phagocytosis, X 19,500 (preparation by Dr. Victor Ferrans as for Fig. 16).

I frequently come back to the thought that it may be that the alpha of Tangier disease may also be its omega. By this I mean that perhaps the still obscure secrets of why we have tonsils and why we have HDL may someday emerge in a single revelation. Surely this possibility would have amused Jiménez-Díaz as the physician, if not as the physiologist.

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